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(54) Title of the invention: Method for extraction or removal of nucleic acid

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Specification

1. Title of the Invention

Method for extraction or removal of nucleic acid.

2. Scope of the Patent Claims

(1) Method for extraction of nucleic acid, wherein, after addition of a protein denaturing agent to a nucleic acid-containing sample, alcohol is added, then the precipitate is collected.

(2) Method for removal of nucleic acid, wherein, after addition of a protein denaturing agent to a nucleic acid-containing sample, alcohol is added, then the precipitate is removed.

(3) Method according to Claim (1), characterized in that the protein denaturing agent is a guanidine salt or urea.

(4) Method according to Claim 1 or 2, characterized in that the alcohol is at least one alcohol selected from ethanol, propanol, butanol, pentanol or hexanol.

3. Detailed Description of Invention

Field of Industrial Application

The present invention concerns a method for extraction or removal of nucleic acid from various nucleic acid-containing samples, such as biological samples.

Prior Art

In recent years, extraction of nucleic acid from biological samples has been widely performed in a variety of fields. For example, the operation of extracting mRNA or DNA from cells producing a desired protein, and also the operation of extracting DNA (or RNA) which is for example to be detected from biological samples in clinical diagnosis wherein viral DNA (or RNA) is detected using DNA probes, are performed in genetic engineering and in the preparation of DNA probes.

Hence, the operation of extracting nucleic acid is very important in a variety of fields.

Previously, for the extraction of nucleic acid, the method of for example adding a caustic reagent to the sample, then performing a phenol or chloroform/phenol extraction 1-3 times, and finally performing an ethanol precipitation, and the method of subjecting cells to the action of a surfactant and proteinase K, then performing a phenol extraction, and then performing an ethanol precipitation, are known. Further, as methods for extracting RNA, the method of adding guanidine thiocyanate to the cells, and performing a centrifugation in a centrifugation solution prepared to a specified density, and the methods of performing ion exchange column chromatography, gel filtration or electrophoresis are known. Moreover, kits or machines for the extraction of nucleic acid from biological samples are known. In these, either the protein is degraded using proteinase K at the same time as the cells are lysed, and the nucleic acid is extracted from the resulting solution using ion exchange column chromatography, or the sample is treated with proteinase K, then a phenol extraction is performed, and then an alcohol precipitation is performed.

Problems of the Prior Art

In methods such as the aforesaid for the extraction of nucleic acid, complex operations are necessary, and apart from the time taken, processing with hazardous solvents is necessary, and also there is a yield problem in that the quantity of nucleic acid obtained is small.

To be specific, in the method wherein a caustic reagent is added to the sample, then a phenol or chloroform/phenol extraction is performed 1-3 times, and finally an ethanol precipitation is performed, time is needed for its implementation because complex extraction operations are necessary, and hazardous alkaline reagents and organic solvents such as chloroform have to be used. In the method wherein a surfactant and proteinase K are used, then a phenol extraction is performed, and then an ethanol precipitation is performed, there is a problem in that time is needed for degradation of the protein by the proteinase K. In the method wherein guanidine thiocyanate is added to the cells, and a centrifugation is performed in a centrifugation solution prepared to a specified density, there is a problem in that the centrifugation operation takes about 1 day. In the methods wherein ion exchange column chromatography, gel filtration or electrophoresis is performed, there is a problem in that the operations are complex and take time to perform. In the methods described above, there are also problems in that because of the complexity of the operations, for example they are very difficult to automate. There are also problems concerning the kits or machines for the extraction of nucleic acid from biological samples, such as that because proteinase K is used, time is needed for it to react.

Means of Solving Problems

The present inventors, as a result of diligent studies concerning nucleic acid extraction methods wherein this can be effected by a simple operation, and complex operations are not necessary, managed to perfect the method of the present invention which has solved the problems seen in the prior art. By means of the present invention, a method for removing nucleic acid from samples is also simultaneously provided. In other words, the present invention is a method for extraction of nucleic acid, wherein, after addition of a protein denaturing agent to a nucleic acid-containing sample, alcohol is added, then the precipitate is collected, and, further it is a method for removing nucleic acid, wherein, after addition of a protein denaturing agent to a nucleic acid-containing sample, alcohol is added, then the precipitate is removed. Below, the invention is described in detail.

The method of the present invention concerns a method wherein nucleic acid such as DNA or RNA is extracted or removed from samples. Nucleic acid in the present specification means DNA and/or RNA, and these may be single-stranded or double-stranded, moreover its genetic properties are immaterial. For example, apart from genomic DNA, the DNA may be DNA

contained in mitochondria or chloroplasts, and the RNA may be messenger RNA or transfer RNA.

Concerning the nucleic acid-containing samples, biological samples such as tissue, cells, blood, bile, pus, spinal fluid, faeces, saliva and sputum, and also can be mentioned as examples. Concerning these samples, if the contained protein and nucleic acid are in a state where they cannot be contacted with the protein denaturing agent and alcohol, in other words if the sample has cell walls or cell membranes or is in an agglomerated state, as necessary homogenizing or surfactant treatment or ultrasonic treatment may for example be performed.

Next, the proteins in the sample are denatured and solubilised by the addition of a protein denaturing agent to the sample. There is no particular restriction as to the denaturing agent, and any can be used provided that it can degrade protein, however, guanidine salts or urea, whose action is outstanding, are ideal. As guanidine salts, for example guanidine thiocyanate, guanidine chloride and the like, and also organic salts of guanidine such as guanidine carbonate, can be used.

The protein denaturing agent can be added to the sample in the solid state (powder, granules) or in the dissolved state, but if it is added in the solid state, a stirring operation should be performed so as to ensure that the protein in the sample is thoroughly denatured.

The protein denaturing agent is added so as to give a concentration capable of denaturing the protein in the sample at the time when it is added to the sample, and such that thereafter the nucleic acid forms a precipitate on addition of the alcohol. For example, if it is guanidine thiocyanate, since adequate denaturation of the protein does not occur below 1.5 M, and adequate precipitation of the nucleic acid on addition of alcohol does not occur above 6.5 M, it is added to the sample so as to give a concentration of about 2.0 M to 6 M, preferably 2.5 M to 5.5 M. If it is guanidine chloride, it can be added so as to give a concentration of 2 M or more, and there is no upper limit. In the case of urea, since adequate denaturation of the protein does not occur at about 4 M, it is added to the sample so as to give a concentration of 4.5 M or more, preferably 6 M or more. The concentrations described above are a general

condition for performing the present invention, and are preferably determined in the light of the amount of protein present in the sample when the present invention is performed.

Although the concentrations of protein denaturing agents are as described above, apart from these, for example the purpose of the present invention is also achieved by firstly adding the protein degrading agent to the sample so as to give a concentration at which the nucleic acid scarcely forms a precipitate on subsequent addition of alcohol, and, during a subsequent alcohol addition, adding a quantity of alcohol sufficient to dilute the degrading agent to a concentration at which the nucleic acid forms a precipitate.

Next, the nucleic acid is precipitated by adding an alcohol to the sample solution containing the protein degrading agent. The alcohols may be exemplified by ethanol, n-propanol, iso-propanol, n-butanol, sec-butanol, tert-butanol, n-amyl alcohol, isoamyl alcohol and tert-amyl alcohol.

The alcohol may be added so as to give a concentration that causes nucleic acid precipitation without causing deposition of the protein denatured by the denaturing agent and present in the solubilized state, but the specific amount added depends on the nature of the alcohol used. With alcohols with longer alkyl chains, in other words, alcohols of higher hydrophobicity, the nucleic acid can be precipitated by the addition of smaller amounts. Hence, when performing the present invention, it is preferable to investigate the ideal amount to add by performing an experiment using the alcohol that is to be used.

Among the specific alcohols stated above that can be used in the present invention, with those other than ethanol and isopropanol, it is known that phase separation from aqueous phases such as buffer solutions occurs. However, in the present invention, if these alcohols are added to a solution containing a guanidine salt, a homogeneous phase is formed, and phase separation does not occur. In the present invention, in order to cause phase separation, the solubility of the alcohol in the said solution may be decreased for example by adding a salt such as common salt.

By means of the above operations, the nucleic acid contained in the sample forms a precipitate, whereas the protein is present in the solution in the dissolved state owing to the

action of the protein denaturing agent. Consequently, after the method of the present invention has been implemented, it is possible to extract or remove the nucleic acid for example by performing a centrifugal separation or membrane separation. Concerning the nucleic acid extracted, there is no restriction whatever for example as regards washing after the present invention with aqueous alcohol solutions and the like not containing the protein denaturing agent. Further, concerning the protein denaturing agent solution after the nucleic acid has been removed according to the present invention, there is no restriction whatever for example as regards decreasing the denaturing agent concentration by performing dialysis and the like after the implementation of the present invention.

Effect of the Invention

The present invention can be implemented without the use of hazardous organic solvents, indeed merely by the operations of adding a protein denaturing agent, and an alcohol to the sample, and subsequent separation or removal of the precipitate. Hence, the time needed for its implementation is also very short, and it has become possible rapidly to perform the operation of nucleic acid extraction or removal for which ½ a day to 2 days were previously necessary. Because of this, it is a particularly useful method in fields such as genetic engineering, where it is necessary to extract nucleic acid from large amounts or large numbers of samples.

Since, as stated above, the present invention can be implemented by very simple operations, it can also be automated.

Further, in the present invention, it is possible to perform all operations in the same reaction vessel; moreover, since it is not necessary to separate or remove the precipitate by column chromatography or the like, sample losses are small, hence it is possible to extract nucleic acids in high yield, or to remove the nucleic acids without loss of protein. The fact that it is possible to perform all operations in the same reaction vessel means that for example if the nucleic acid to be extracted or removed is hazardous viral DNA or the like, and the sample is cells infected with the said virus, it is possible to minimize contamination with these.

Practical Examples

Below, in order to explain the present invention in more detail, practical examples are given, however these practical examples are an illustration, and do not limit the present invention.

Practical Example 1

K562 cells, which are human leucocyte cancer cells (these cells are well-known and can be purchased for example from Dai-Nippon Pharmaceutical (Ltd.)) were cultured in PRMI-1640 culture medium containing 1 % foetal bovine serum. On attainment of 500,000 cells per ml of culture suspension, 1 ml of suspension was collected in a sampling tube, and the cells in the precipitate were recovered by centrifuging for 5 minutes at 500 rpm.

After addition of 0.4 ml of 10 mM Tris hydrochloride buffer solution (pH 8.0) containing 5 M guanidine thiocyanate and 1 mM EDTA to the cells, the mixture was stirred for 1 minute at room temperature, and then 1 ml of 99.5% ethanol was added. Next, this solution was centrifuged for 5 minutes at 15,000 rpm, and the nucleic acid was extracted in the precipitate.

The supernatant was discarded, and after addition of 70% ethanol to the precipitate and washing, the nucleic acid was obtained by drying.

As a result of the action of restriction enzymes (Bam HI, Eco RI, Alu I), RNase and DNase on the nucleic acid extracted as above, it was found that the reactions due to these enzymes were not inhibited; further, replication with DNA polymerase using the extracted nucleic acid as the template was also not inhibited. These results show that restriction enzyme inhibiting substances such as histones have been removed.

Practical Example 2

K562 cells which are human leucocyte cancer cells were cultured in the same way as in Practical Example 1. On attainment of 500,000 cells per ml of culture suspension, 1 ml of suspension was collected in a sampling tube, and the cells in the suspension were recovered by centrifuging for 5 minutes at 500 rpm.

After the centrifugation, the supernatant was discarded, and after addition of 0.4 ml of 10 mM Tris hydrochloride buffer solution (pH 8.0) containing 5 M guanidine thiocyanate and 1 mM

EDTA to the cells, the mixture was stirred for 1 minute at room temperature, and then 1 ml of 99.5% ethanol was added. Next, the nucleic acid was recovered by filtering this solution with a tetrafluoroethylene membrane (Norton Ltd, ZITEX, pore diameter = midum).

After the filtration, this membrane was washed with 70% ethanol and the nucleic acid in the buffer solution was obtained by drying.

When the same tests as in Practical Example 1 were performed on the nucleic acid extracted as above, all the reactions were achieved with no inhibition.

Practical Example 3

E. coli JM109 strain transformed with the plasmid pIBI176 (IBI Ltd.) was cultured in LB culture medium.

On attainment of 10^8 cells per ml of culture suspension, 0.1 ml of this was collected in a sampling tube, and after addition of 0.3 ml of 8 M guanidine chloride, it was stirred for 1 minute. After stirring, 0.2 ml of 99.5 % isopropanol were added, and after stirring the nucleic acid was extracted by centrifuging for 5 minutes at 15,000 rpm.

The supernatant was discarded, and the precipitate was washed twice with 70% ethanol, then dissolved in 0.4 ml of 10 mM Tris hydrochloride buffer (pH 8.0) containing 1 mM EDTA, and after centrifuging for 5 minutes at 15,000 rpm, the impurities were removed.

1 ml of 99.5% ethanol was added to the supernatant obtained as aforesaid, the precipitate obtained by centrifugation for 5 minutes at 15,000 rpm was dried, and when the same tests as in Practical Example 1 were performed, it was found that all the enzyme reactions were achieved with no inhibition.

Practical Example 4

K562 cells, which are human leucocyte cancer cells, were cultured in LB culture medium. On attainment of 500,000 cells per ml of culture suspension, 1 ml of suspension was collected in a sampling tube, and the cells were recovered by centrifuging for 5 minutes at 500 rpm.

After addition of 0.4 ml of 10 mM Tris hydrochloride buffer solution (pH 8.0) containing 8 M urea and 1 mM EDTA to the precipitate, the mixture was stirred for 1 minute at room temperature, and then 1 ml of 99% secondary butyl alcohol was added. Next, this solution was filtered with a similar tetrafluoroethylene membrane to that used in Practical Example 2. After the filtration, this membrane was washed with 70% ethanol and the nucleic acid in the buffer solution was obtained by drying.

When the same tests as in Practical Example 1 were performed on the nucleic acid extracted as above, all the reactions were achieved with no inhibition.

Practical Example 5

BLV (bovine leucocyte virus: Virology, Vol.138, p.82, 1984) DNA was integrated into M13 phage (Takara Ltd.) at a Bam HI site. For reference, a part of the BLV DNA is shown in Figure 1.

The M13 phage having 1 ng of DNA was added to 100 μl of bovine serum, 300 μl of 6 M guanidine isocyanate were added to this serum, and the mixture was stirred for 1 minute.

After addition of 10 μl of 10 mg/ml salmon DNA as carrier and 1 ml of ethanol to this solution, it was stirred for 1 minute, and centrifuged for 5 minutes at 15,000 rpm. The supernatant was discarded, and the precipitate was dried, and dissolved in 50 μl of 10 mM Tris hydrochloride buffer solution (pH 8.0) containing 1 mM EDTA.

Synthetic DNA having the complementary sequence to the aforesaid base sequence made up of the base sequence shown in Figure 2 was prepared using a DNA synthesizer (Applied Bio-systems Ltd.). The 5' terminus of the DNA prepared was aminated, and alkaline phosphatase was bonded to it. The aforesaid sequence was detected by the dot blotting method using this DNA as the probe.

With the M13 phage DNA, a coloration was observed, but with the bovine serum used as a control no coloration whatever was observed. This shows that, it is possible to detect minute amounts of DNA (BLV DNA) from highly concentrated protein solutions (bovine serum) by means of the present invention.

4. Brief Description of the Diagrams

Figure 1 is a diagram showing the part of the BLV DNA used in Practical Example 5 of the present invention. The symbols in the diagram mean the same as the symbols used in normal genetic engineering. Further, in the diagram, the regions underlined indicate the regions which specifically pair with the DNA shown in Figure 2.

Figure 2 shows the DNA base sequence which specifically pairs with the underlined regions in the DNA used in Practical Example 5 of the present invention, which is shown in Figure 1.

Patent Applicant Tosch K.K.

Fig.1-1

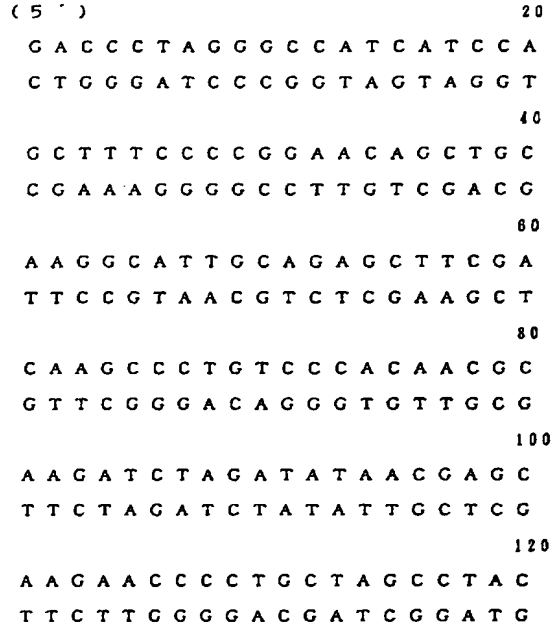

Fig.1-2

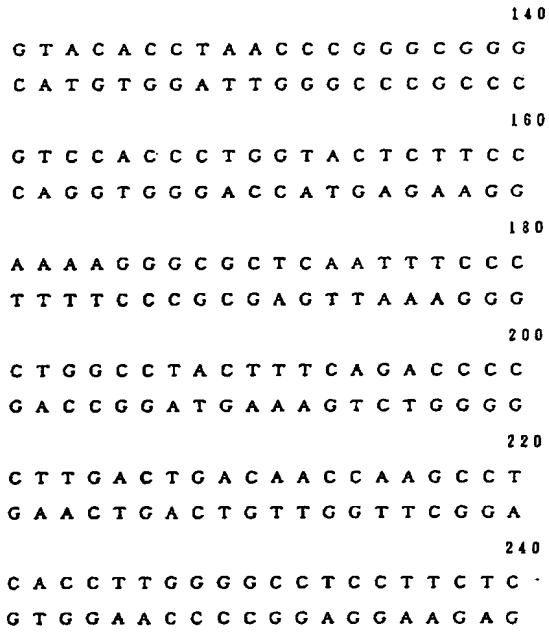

Fig.1-3

Fig.2